53BP1 and the LINC Complex Promote Microtubule-Dependent DSB Mobility and DNA Repair

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell lines, cell treatments, plasmids, and shRNAs

SV40LT TRF2^F/F^ Cre-ER^T1^, TRF2^F/F^ 53BP1^-/^- and TRF2^F/F^ 53BP1^-/^- Lig4^-/^-, TRF2^F/F^ Rif1^F/F^ MEFs have been either previously described (Dimitrova et al., 2008; Denchi and de Lange, 2007; Lottersberger et al., 2013; Zimmermann et al., 2013) or were obtained by intercrosses with Cre-ER^T1^ mice (TRF2^F/F^ 53BP1^-/^- Cre-ER^T1^ and TRF2^F/F^ 53BP1^-/^- Lig4^-/^- Cre-ER^T1^). SUN1^-/^- and SUN2^-/^- mice (012715 and 012716, The Jackson Laboratory), PtIP^F/+^ mice (019143, The Jackson Laboratory), and Rif3A^F/F^ mice (gift from P. Igarashi and L.S.W. Goldstein; (Lin et al., 2003)) were used to derive all compound genotypes by standard crosses with TRF2^F/F^, 53BP1^+/-^, Lig4^+/-^, and Rif1^F/F^ mice. All MEFs were isolated from E12.5 embryos. Genotyping was done by Transnetyx Inc. using real time PCR with allele-specific probes. MEFs were immortalized with pBabeSV40LargeT (a gift from G. Hannon) at P2 or 3 and cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Cellgro) supplemented with 15% fetal bovine serum (FBS) (Gibco), non-essential amino acids (Gibco), L-glutamine (Gibco), penicillin/streptomycin (Gibco), 50 μM β-mercaptoethanol (Sigma). Cre was induced with 0.5-1 μM 4-OHT or with three infections at 12 h intervals with pMMP Hit&Run Cre retrovirus derived from transfected Phoenix cells as previously described (Celli and de Lange, 2005). For timed experiments, time point 0 was set at the time of addition of 4-OHT or at 12 h after the first Hit&Run Cre infection. For expression of mCherry-53BP1-2-pWZL, EGFP-TRF1-pWZL (Dimitrova et al., 2008), YFP-tubulin ((Beaudouin et al., 2002); subcloned in pLPC), mAG-hGeminin (Davoli et al., 2010), and mutated alleles of 53BP1DB-pMX, 20 μg of plasmid DNA was transfected into Phoenix cells using CaPO4 precipitation as previously described (Wu et al., 2012). The retroviral supernatant was used for six infections at 6-12 h intervals. Cells were selected for 3-5 d in 2-3 μM Hygromycin or 2-6 μM Puromycin. ShRNAs for
Nesprin 4, Kif3A and Kif5B (TRCN0000-195990/184115/090405/090407/106535/091481 Opensysstem) were introduced with three infections/day (6-12 h intervals) over two days using the pLKO.1 lentiviral vector (Openbiosystem) produced in 293T cells and infected cells were selected for 3-5 d in Puromycin. Taxol (Paclitaxel, Sigma), Nocodazole (M1404, Sigma), and PARP inhibitor (Opalarib, AZD2281, Selleck chemicals) were dissolved in DMSO and added at a final concentration of 20 μM, 1 μg/ml, and 2 μM, respectively.

For the survival assay, BRCA1 shRNA infected cells were plated in a 6-well plate, in duplicate at 10, 100, and 1000 cells per well. After 24 hours, cells were either left untreated or treated with PARP inhibitor at various concentrations and incubated for 7 days. Media was changed after 4 days. After washing with PBS, colonies were fixed and stained for 1 min in a solution containing 50% methanol, 2% Methylene Blue and rinsed with water. Colony numbers were determined using wells with 10-40 colonies and the % survival at each PARPi concentration compared to the untreated cells was calculated.

**Immunoblotting**

Immunoblotting was performed as previously described (Celli and de Lange, 2005) with minor modifications. Cells were lysed in 2X Laemmli buffer at 5X10^3 cell/μl and the lysate was denatured for 10 min at 95°C before shearing with an insulin needle. Lysate equivalent to 10^5 cells was resolved using SDS/PAGE and transferred to a nitrocellulose membrane. The following primary antibodies were utilized: TRF2 (1254, rabbit polyclonal); Chk2 (BD 611570; BD Biosciences); 53BP1(100-304A; Novus Biologicals) or (ab175933; ABCAM), human 53BP1 (BD 612522; BD Biosciences), Myc (9B11, mAb 2276; Cell Signaling), SUN1 (ab74758; Abcam), SUN2 (ab87036; Abcam); Kif5B (ab
Live-cell imaging

Dysfunctional telomeres were visualized using mCherry-BP1-2 (h53BP1, 1220-1711aa) as described previously (Dimitrova et al., 2008). Cre-treated TRF2<sup>−/−</sup> cells were plated onto MatTek glass bottom plates and grown for 2 days before imaging. An hour before imaging, cells were changed into Leibovitz’s L-15 medium (Gibco) supplemented with 15% FBS, non-essential amino acids, L-glutamine, penicillin/streptomycin and 50 μM β-mercaptoethanol, and allowed to equilibrate for one hour. For the analysis of irradiation-induced DSBs, cells were plated 1-2 days before imaging, changed into imaging medium, irradiated and let recover for one hour. Imaging was done at 37°C using an environmental chamber using a DeltaVision RT microscope system (Applied Precision) with a PlanApo 60x 1.40 NA objective lens (Olympus America, Inc.). 5 μm Z-stacks at 0.5 μm steps were acquired using SoftWoRx software with 50 msec exposure time, every 30 sec over 10 min (t=20 frames) at 2 x 2 binning with 512 x 512 pixels in final size. Images were deconvolved and 2D-maximum intensity projection images were obtained using SoftWoRx software. Tracking of mCherry-BP1-2 foci was performed with ImageJ software for at least 10 cells per condition. Cells were registered by the StackReg plugin using Rigid Body (Thevenaz et al., 1998). Next, particles were detected and tracked using the Mosaic Particle Detector and Tracker plugin (Sbalzarini and Koumoutsakos, 2005) with the following parameters: radius=1-2 pixels; cutoff=1-2 pixels; percentile=1-6; link range=1; displacement=5 pixels. The x and y coordinates of each trajectory were output for further calculation. Per cell, all mCherry-BP1-2 foci that were continuously tracked for at least 19 of the 20 frames were analyzed. The analysis of the eGFP-TRF1-marked telomeres was similarly conducted using the following parameters:
radius=1 pixels; cutoff=1 pixels; percentile=8-12; link range=1; displacement=5 pixels.

**Identification of distorted nuclei**

The average x and y values of all the foci was calculated in each frame as the Geometrical Center (GC) and normalized over the \( GC_{t=0} \). The distance traveled by the GC between each time points \( t=b \) and \( t=a \) was calculated as Movement of Geometrical Center

\[
MGC_{b-a} = \sqrt{(x_{GC}^{t=b} - x_{GC}^{t=a})^2 + (y_{GC}^{t=b} - y_{GC}^{t=a})^2}
\]

and the maximal MGC (MMGC) for each cell was identified. Cells were discarded if their MMGC exceeded the arbitrary threshold of 2, or if their MMGC exceeded the secondary threshold of 1 and another parameter was also above threshold.

The Difference of the Average Distances of all the \( i \) foci in the cell and \( GC_{t=0} \) \((\Delta AD)\) between each time points \( t=b \) and \( t=a \) was calculated as

\[
AD_{b-a} = \left( \frac{\sum_{j=1}^{n} \sqrt{(x_{i=t=b}^{j} x_{i=t=0}^{GC})^2 + (y_{i=t=b}^{j} y_{i=t=0}^{GC})^2}}{n} \right) \left( \frac{\sum_{j=1}^{n} \sqrt{(x_{i=t=a}^{j} x_{i=t=0}^{GC})^2 + (y_{i=t=a}^{j} y_{i=t=0}^{GC})^2}}{n} \right)
\]

and the maximal \( \Delta AD \) (M\( \Delta AD \)) for each cell was identified. Cells were discarded if M\( \Delta AD \) exceeded the arbitrary threshold of 2, or if M\( \Delta AD \) exceeded the secondary threshold of 1 and another parameter was also above threshold.

Finally, the trajectories travelled by each focus \( i \) per cell, relatively to the GC, were normalized to the coordinates \( x_{i=t=0}^{j} \) and \( y_{i=t=0}^{j} \) and projected together on a XY plane. The percentage of foci in each quadrant was calculated for each time frame: Upper Right (UR(\( \% \))), Lower Right (LR(\( \% \))), Upper Left (UL(\( \% \))), Lower Left (LL(\( \% \))) and the average of these values during the time-lapse was derived. Laterality (LAT (\( \% \))),
Verticality (VER (%)) and Diagonality (DIA (%)) were calculated for each time frame as:

\[
LAT(\%) = \left\{ \begin{array}{ll}
((UR(\%)+LR(\%)) & 0.5) \\
100 & 0.5
\end{array} \right. \times 100
\]

\[
VER(\%) = \left\{ \begin{array}{ll}
((UR(\%)+UL(\%)) & 0.5) \\
100 & 0.5
\end{array} \right. \times 100
\]

\[
DIA(\%) = \left\{ \begin{array}{ll}
((UR(\%)+LL(\%)) & 0.5) \\
100 & 0.5
\end{array} \right. \times 100
\]

and the average of these values during the time-lapse were derived. Cells were discarded if UR, LR, UL, LL, LAT, VER or DIA exceeded the arbitrary threshold of 40%, or if they exceeded the secondary threshold of 30% and another parameter was also above threshold.

The Cumulative Distance traveled in 10 min by each of the foci \(i\) (CD\(i\)) was calculated relative to the GC, as previously described (Dimitrova et al., 2008), as

\[
CD_i = \frac{20}{\sum_{t=1}^{n} D_i(t)^2}
\]

Mean Square Displacement (MSD) was calculated as

\[
MSD(t) = \frac{1}{n} \sum_{i=1}^{n} D_i(t)^2,
\]

where

\[
D_i(t) = \sqrt{\left((x^i_t - x^{GC}_t)^2 + (y^i_t - y^{GC}_t)^2\right) + \left((x^i_{t-1} - x^{GC}_{t-1})^2 + (y^i_{t-1} - y^{GC}_{t-1})^2\right)}.
\]

All data output in pixels (standard ImageJ output) were converted to meters by the formula, 1 pixel = 0.215 μm, based on the characteristics of the objective.

Diffusion Coefficient \(D\) was calculated as

\[
D = m/4
\]

where \(m\) is the slope of the MSD after fitting to a linear curve.

Anomalous Diffusion Coefficient \(\alpha\) was derive using MATLAB by the fitting of MSD to the diffusion model function:
\[ MSD = A + t \]

For cumulative distance, statistical analysis was performed using Prism Software applying the Mann-Whitney test.

**IF and IF-FISH**

Cells grown on coverslips were fixed for 10 min in 3% paraformaldehyde/2% sucrose at room temperature. IF and IF-FISH were then carried out as previously described (Takai et al., 2003; Dimitrova et al., 2008). Digital images were captured on a Zeiss Axioplan II microscope with a Hamamatsu C4742-95 camera using Volocity software. The following primary antibodies were utilized: 53BP1(100-304A; Novus Biologicals, or ab175933; Abcam), human 53BP1 (BD 612522; BD Biosciences), γH2AX (JBW301; Millipore), Rif1 (1240, rabbit polyclonal; (Buonomo et al., 2009)).

**Telomere ChIP**

Telomeric ChIP was conducted as previously described (Loayza and de Lange, 2003). The following primary antibodies were utilized: TRF1 (1449, crude serum), 53BP1(ab175933; ABCAM), SUN1 (ab74758; Abcam), SUN2 (ab87036; Abcam).

**Analysis of metaphase chromosomes**

Telomeres were detected by FISH on metaphase spreads using a previously described protocol (Lansdorp et al., 1996) with minor modifications (Celli and de Lange, 2005; Doksani et al., 2013). Images were acquired using a Zeiss Axioplan II microscope with a Hamamatsu C4742-95 camera using Volocity software. For radial chromosomes, MEFs were incubated with PARPi (2 μM) for 16 h before harvest and the trypsinized cells were incubated in 0.055 M KCl at 37°C for 30 min before being processed.
In-gel analysis of single-stranded telomeric DNA

Mouse telomeric DNA was analyzed on CHEF gels as described previously (Wu et al., 2012).

qRT-PCR Analysis

Nesprin-4 RNA levels were analyzed after RNA isolation and quantitative PCR as described in (Kabir et al., 2014). Differences calculated using the ΔCT method were normalized to GAPDH expression. Primers:

Nesprin-4_FW: TAGCCTGGTGCTTGAGAAGG
Nesprin-4_RV: AGGAGTGGAAGGTACTGG
GAPDH_FW: GTGTTTCCTACCCCCCAATGTGT
GAPDH_RV: ATTGTCACTACCAGAAATGAGCTT

53BP1 alleles and PTIP construct

S/TQ mutants were generated using Gibson cloning using previously published constructs as a template (Bothmer et al., 2011). Briefly, the mutated residues for 53BP1ΔPTIP (S6A, S13A, S25A, S29A) and 53BP1ΔMOB (S674A, T696A, S698A, S784A, S831A, T855A, S892A, S1068A, S1086A, S1104A, T1148A, S1171A, S1219A) were obtained by PCR using 53BP1-28A as a template. The non-mutated residues and vector backbone were generated by PCR using 53BP1DB as the template. To generate the full-length vector, both PCR products were joined using Gibson cloning based on homology from the PCR primers. Primers:

53BP1ΔPTIP_FW: GAAAAACAAGGTTGCAGACCCTGAGTTGATTTCTC;
53BP1ΔPTIP_RV: GAAGAATCCACAGGGTCTGCAACCTTGTTTTC;
53BP1ΔMOB_FW: CAGTTCCGTACCAGCTACTCGATCTGAGGCAC;
53BP1ΔMOB_RV: GTGCCTCAGATCGAGTAGCTGGTGACGGAACTG;
53BP1Nterm_FW: GGTGGACCATCCTCTAGACTGCGGATCCGAATTC;
53BP1Nterm_RV: GAATTC

Full-length mouse PTIP was cloned as a BamHI/EcoRI fragment into the pLPC retroviral vector, adding a myc tag to the C terminus.

**Coimmunoprecipitation**

4-5x10^6 293T cells were plated in a 10 cm dish 20-24 h prior to transfection by CaPO_4 precipitation using 10 µg of each plasmid DNA as indicated. Medium was changed 12 hr after transfection and 24-28 h later cells were incubated with zeocin for 20 min, harvested by scraping in cold PBS on ice, and collected by centrifugation. After resuspension in 0.5 ml hypotonic lysis buffer (10% glycerol, 10mM KCl, 10 mM Hepes (pH 7.9), 0.1 mM EDTA, 0.5% NP-40, 1.5 mM MgCl_2, Complete protease inhibitor mix (Roche), and PhosSTOP phosphatase inhibitor mix (Roche)), the KCl concentration was raised to 400 mM. Samples were sonicated for 1 min in water bath solicitor and equal amount of lysis buffer, without KCl, was added to reduce KCl concentration to 200 mM. After centrifugation at 16,000 rpm for 10 min at 4°C, samples were incubated with magnetic beads conjugated with anti-FLAG M2 antibody (Sigma) as described (Subbotin and Chait, 2014) and nated at 4°C for 1 hr. Beads were washed 7 times with the lysis buffer containing 150 mM KCl and immunoprecipitated proteins were eluted with 50 µl of 2xLaemmli buffer. Samples were boiled for 5 min before separation on SDS-PAGE.
SUPPLEMENTAL REFERENCES


